

## MANIPULATION OF MIN GENES IN PLANTS

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from provisional patent application Serial No. 60/130,403 filed April 19, 1999.

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STATEMENT REGARDING FEDERALLY  
SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

## BACKGROUND OF THE INVENTION

10 The modern agricultural industry has devoted considerable resources toward the development of phenotypically distinct plants with economically advantageous qualities. Valuable features in food crops include increased vigor, disease resistance, greater yields, extended shelf-life, and enhanced nutritional content.

15 The development of high yielding food crops is particularly important. Each year, the tillable land available for agricultural production is reduced as more acreage is devoted to alternative uses. At the same time, the human population is rapidly increasing. Therefore, it is essential to increase agricultural productivity in order to meet the nutritional needs of the world's burgeoning population.

20 Efforts to develop crop plants that produce higher yields have been directed toward pest control, or toward the selection and breeding of varieties that bear greater numbers of fruit, or that produce larger fruit. These crop breeding endeavors are very time-consuming and labor-intensive, but have historically increased crop yields incrementally over time. Modern techniques of recombinant DNA manipulation and genetic engineering offer the prospect of the more rapid creation of new plant varieties with novel traits. The creation of genetically modified, or transgenic, plants with altered  
25 phenotypes arising from artificially inserted genetic constructions has become a

common practice in modern agriculture.

If one is going to genetically engineer plants, the genetic engineering or recombinant DNA manipulation of plastids is one area in which improvements to plants might be targeted. Plastids are membrane-delimited organelles in plant cells which are essential for sustaining plant growth and cell viability. They are the site for the synthesis of essential amino acids, vitamin E, pro-vitamin A, starch, certain growth hormones, lipids, and pigments such as carotenes, xanthophylls, and chlorophylls. In plants, plastids include chloroplasts, chromoplasts, leucoplasts and amyloplasts, which are typically found in all organs of the plant including its leaves, roots, stems, petals, and seeds.

The specialized plastid chloroplast is where photosynthesis occurs. Photosynthesis in plants is an important biosynthetic process upon which virtually all living organisms depend for our very existence. During photosynthesis, energy in the form of light is converted to ATP, which fuels a series of enzymatic reactions that catalyze the synthesis of carbohydrates, which are further used for metabolic energy in the plant. Photosynthesis also produces molecular oxygen ( $O_2$ ) as a byproduct. Because photosynthesis is the source of metabolic energy in plants, photosynthetic efficiency is a significant factor associated with general plant growth and vigor. Chloroplasts also synthesize amino acids and lipids.

U.S. Patent 5,981,836, incorporated herein by reference, discloses genetic constructs capable of altering the number and size of plastids in plant cells. These constructs contain an *Arabidopsis* plastid division FtsZ protein coding sequence and a promoter, not natively found associated with the FtsZ protein coding sequence, which promotes expression of the *Arabidopsis* plastid division FtsZ protein coding sequence in the plant. The FtsZ protein is a bacterial cytoskeletal protein and structural homologue of tubulin that polymerizes on the inner surface of the cytoplasmic membrane to form a cytokinetic ring during cell division. Transgenic expression of the coding sequence results in a high percentage of novel phenotypes characterized by alterations in the number and size of plastids in the cells of the plant in which the construct is expressed.

There is also on-going efforts to make transgenic plants that are more suited for particular applications or which have transgenes inserted into them to have localized

effects inside the cells of the plants. For example, there are a number of transgenes inserted into plants which maximize the usefulness of the inserted traits if the transgenes are transformed into the chloroplasts of the plant. Since one method for chloroplast transformation is based on the delivery of transgenes coated onto small carrier particles  
5 into the interior of the chloroplasts themselves, this technique is easier to perform if the chloroplasts themselves are larger than normal. So one technique that would be useful for this effort is to make plants with larger chloroplasts.

What is needed in the art are additional means for altering the shape, size and/or number of chloroplasts and other plastids in agronomically and horticulturally important  
10 plants to achieve greater plant productivity and nutritional quality.

### BRIEF SUMMARY OF THE INVENTION

The present invention is a plant comprising in its genome a genetic construction including a sense or antisense MinD protein coding sequence and a promoter, not  
natively associated with the MinD protein coding sequence, which promotes expression  
15 of the sequences in the plant, wherein expression of the sequence in the plant causes alterations in the number, shape and/or size of the plastids in the plant cells of the plant. The present invention also discloses a method for altering the number, shape and/or size of the plastids using the genetic construct described above.

The present invention also includes DNA sequences (SEQ ID NO:1 and SEQ ID  
20 NO:3) representing genes that function in regulating plastid division, and which, when ectopically expressed, alters the number, shape and/or size of chloroplasts and other types of plastids present in plant cells.

The present invention is also directed toward a genetic construct including a MinD protein coding sequence and a promoter that promotes expression of the sequence  
25 in plants, the promoter not being natively associated with the MinD protein coding sequence.

The present invention is also a seed, including in its genome a genetic construct comprising a MinD protein coding sequence and a promoter, not natively associated with the MinD protein coding sequence, that promotes gene expression in plants.

30 The present invention is also a plant cell including in its genome a genetic

construct comprising a MinD protein coding sequence and a promoter, not natively associated with the MinD protein coding sequence, that promotes gene expression in plants.

It is an object of the present invention to provide a transgenic plant that has a novel phenotype with advantageous qualities, including decreased numbers of enlarged chloroplasts.

Other objects, advantages, and features of the present invention will become apparent after review of the specification and drawings.

#### BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1 is an illustration of the alignment of MinD proteins from several photosynthetic organisms.

Fig. 2 is a model showing the proposed effect of plastid-localized *AtMinD1* on the positioning of the plastid division apparatus.

Fig. 3 are graphs illustrating the frequency distribution of chloroplast sizes in mesophyll cells from *Arabidopsis* wild-type plants and *Arabidopsis* plants transformed by an antisense *AtMinD1* construct.

#### DETAILED DESCRIPTION OF THE INVENTION

It is disclosed here that nuclear-encoded, plastid-targeted forms of genes encoding a MinD protein have been identified in plants, and these genes have been shown to play an important role in the division of plastids. The MinD genes had previously only been demonstrated to exist in prokaryotes. The data presented here demonstrates that all plants natively have MinD genes, and that the function of the endogenous genes can be altered by genetic engineering.. Reduced expression of an endogenous MinD gene in a transgenic plant results in asymmetric plastid division leading to an abnormally heterogeneous distribution of plastid numbers and sizes in plant cells. Overexpression of the MinD gene in a transgenic plant results in the inhibition of plastid division and fewer numbers of large plastids.

One aspect of the present invention is a plant that contains in its genome a genetic construct having a sense or antisense plant MinD protein coding sequence and a

promoter, not natively associated with the MinD protein coding sequence, which promotes expression of the sequence in plant cells. Insertion of the genetic construction results in plants having a high percentage of novel phenotypes characterized by alterations in the number, shape and/or size of plastids in cells of the plant in which the  
5 construct is expressed.

The identification and characterization of two initial MinD coding sequences from plants that are useful in the present invention are described in the examples below. The sequence designated AtMinD (At for *Arabidopsis thaliana*, SEQ ID NO:1) was identified by BLAST similarity searching on the basis of homology to bacterial MinD  
10 genes. The sequence designated TeMinD (Te for *Tagetes erecta*, SEQ ID NO:3) was identified by cDNA library screening on the basis of homology to bacterial MinD genes.

It should be understood that the initial plant MinD genes, the identification of which are described here, were identified based on sequence comparison to the analogous genes known in bacteria. Since the bacterial gene sequence was sufficient to  
15 permit the identification of plant MinD genes, and since the plant MinD will be more closely related to each other than they are to bacterial genes, the data presented here make possible the recovery of the respective MinD gene from most, if not all, plant species.

Based on analogous function to the bacterial proteins, plant MinD proteins are  
20 involved in the placement of the FtsZ ring during plastid division. The plant MinD genes are believed to descend from a key prokaryotic cell division mechanism through the evolution of photosynthetic eukaryotes. The process of cell division in bacteria is mediated by a set of at least ten proteins that assemble into a macromolecular complex at the cell midpoint. Chief among these is the bacterial cell division protein FtsZ, a  
25 prokaryotic cytoskeletal protein and structural homologue of tubulin that polymerizes on the inner surface of the cytoplasmic membrane to forms a contractile ring. Assembly of the FtsZ ring is the earliest known step in formation of the bacterial division complex.

The mechanism by which placement of the FtsZ ring is determined in bacteria is still uncertain, but genetic studies have uncovered some of the critical players. In *E.*  
30 *coli*, precise localization of the FtsZ ring at the cell center is established by the Min system of proteins, comprising MinC, MinD, and MinE. In mutants lacking MinC or

MinD, the FtsZ ring is frequently misplaced near one of the cell poles such that cell division results in the formation of nonviable "minicells" which lack chromosomes and cannot expand. Thus, MinC and MinD act in wild type cells by inhibiting FtsZ ring formation at polar sites and restricting the ring to the midcell. This activity in *E. coli* involves an oscillation of both MinC and MinD from one cell pole to the other. MinE, which is targeted independently of FtsZ to a medial ring, prevents MinC and MinD from localizing at the midcell, thereby allowing the FtsZ ring to assemble specifically at this position.

In *Bacillus subtilis*, MinC and MinD also prevent FtsZ ring assembly at polar sites, but are localized at both poles simultaneously and do not oscillate. *B. subtilis* lacks MinE, relying instead on a different protein, DivVIA, to tether MinC and MinD to the cell poles. Though the mechanisms restricting the activity of MinC and MinD to polar sites are different in *E. coli* and *B. subtilis*, in both cases the absence of these proteins at the midcell establishes the site of FtsZ ring assembly, and MinD is required for the proper localization and division-inhibiting activity of MinC.

In plants and other photosynthetic eukaryotes, constriction of the chloroplast during division usually occurs at the middle of the plastid perpendicular to the long axis. See the diagram of Figure 2. These observations indicate that the positioning of the plastid division machinery in plants, like the positioning of the FtsZ ring in bacteria, is a carefully regulated process. The discovery of the existence of a nuclear gene from *Arabidopsis* and *Tagetes* encoding a chloroplast-targeted homologue of MinD and the examination of the relationship between chloroplast shape, size and number in transgenic plants indicates that a Min-based system operates in specifying placement of the plastid division components in plant cells during plastid division.

As used herein, "MinD" refers to the *Arabidopsis* MinD protein coding sequence (SEQ ID NO: 2) and the *Tagetes erecta* (Marigold) MinD protein coding sequence (SEQ ID NO: 4), as well as the analogous gene sequences from other plants as well as variations and mutants thereof which retain plastid division control functionality. As shown in Figure 1, the MinD proteins are highly conserved among diverse species capable of conducting photosynthesis. It is expected that all plants contain MinD genes homologous to the *Arabidopsis* and *Tagetes* genes. The bacterial MinD protein is also

homologous to the plant MinD genes and can be used as well in transgenic plants.

Given the apparent ubiquitousness and high degree of conservation of the MinD proteins among plant species, it is reasonable to expect that MinD genes, of which the AtMinD and the TeMinD genes are but two examples, from any plant could be used in the

5 practice of the present invention. For example, MinD genes from plants that are raised for their agricultural or horticultural value may be used in the practice of the present invention. It can be expected, from the sequence data presented below, that any plant MinD have at least 50%, and more likely at least 80%, sequence identity at the amino acid level with either the *Arabidopsis* or the *Tagetes* MinD protein sequence. The  
10 *Arabidopsis* and *Tagetes* MinD sequences compared to each other have a sequence identity at the amino acid level of 92%, a high degree of sequence identity. By sequence identity it is meant that at least the defined percentage of amino acid residues in the proteins being compared have the same identity and are located in the same sequence order as the corresponding amino acids in the protein to which it is being compared. A  
15 useful calculation (as used here) for amino acid sequence identity comparison is done using pairwise comparisons using the SIM local alignment algorithm (Huang and Miller (1991)) with the default parameters specified on the ExPASy Molecular Biology Server, Swiss Institute of BioInformatics. The comparison at the amino acid level means that two genes being compared may have nucleotide sequences that differ more greatly than  
20 the amino acid sequence, which is possible given the degeneracy of the genetic code, but that they encode amino acids which have the requisite degree of sequence identity.

It is specifically contemplated that any MinD protein coding sequence could be used in the practice of the present invention. "MinD protein coding sequence" is defined to include any plant DNA sequence capable of overexpressing or reducing the  
25 activity of the MinD gene native to the plant in which the MinD protein coding sequence is introduced. A MinD protein coding sequence may be an unmodified genomic entire gene sequence isolated from any plant, a cDNA sequence derived from any plant, a genomic or cDNA sequence that is modified to contain minor nucleotide additions, deletions, or substitutions, or a synthetic DNA sequence. The term is  
30 intended to apply, as well, to analogous sequences from other plants as well as allelic variations and mutations which are still capable of controlling plastid division.

By "plastid division activity" it is meant the ability to cause alterations in the number or size of the chloroplast or other types of plastids present in cells of a transgenic plant in which the MinD protein coding sequence is expressed.

By "transgene" it is meant to describe an artificial genetic construction carried in the genome of a plant and inserted in the plant or its ancestor by gene transfer. Such transgenes are transmissible by normal Mendelian inheritance once inserted into the genome of a parental plant.

It is specifically envisioned that transgenic plants can be made with a transgene for a MinD protein coding sequence which selectively either up-regulates or down-regulates plastid MinD division activity. For fewer plastid divisions, extra copies or high expression copies of MinD protein coding sequence transgenes are inserted into plants, resulting in fewer and larger plastids in the transgenic plants. Sometimes high expressing plants will produce only one or a very few chloroplasts per cell. For more plastid division activity, the use of an antisense MinD protein coding sequence transgene, or any other gene inhibition technique, may be used to down regulate plastid division activity resulting in a greater variability in plastid number and size. Both up and down regulation of plastids will be useful for certain applications.

Transgenic *Arabidopsis* plants were obtained as a model system using the *Agrobacterium* transformation system, as described in the examples. *Arabidopsis* is often used as a model plant in such experiments because of the relatively small size of its genome and also because it is a small compact plant easy to grow and easy to conduct experiments on. *Agrobacterium*-mediated transformation is used since it is known to work well with many dicot plants and some monocots. Other methods of transformation equally useful in dicots and monocots may also be used in the practice of the present invention. Transgenic plants may be obtained by particle bombardment, electroporation, or by any other method of transforming plants known to one skilled in the art of plant molecular biology. The experience to date in the technology of plant genetic engineering is that the method of gene introduction is not of particular importance in the phenotype achieved in the transgenic plant.

The present invention is also directed toward a genetic construct comprising a MinD protein coding sequence and a promoter, not natively associated with the



sequence, which promotes expression of the MinD protein coding sequence in plants at levels sufficient to cause novel phenotypes. The construct may contain the sequence in either the sense or antisense orientation. The development of constructs that have been found to alter the number or size of chloroplasts in transformed plant cells is described  
5 in the examples below. Briefly, relevant features of these constructs include a kanamycin resistance marker and, in 5' to 3' order, the CamV 35S promoter operably connected to a chloroplast division sequence, and a transcriptional terminator, or polyadenylation sequence, from an *Agrobacterium* gene known as OCS.

The CaMV 35S promoter is a constitutive promoter known to function in a wide  
10 variety of plants. Other promoters that are functional in the plant into which the construct will be introduced may be used to create genetic constructs to be used in the practice of the present invention. These may include other constitutive promoters, tissue-specific promoters, developmental stage-specific promoters, and inducible promoters. Promoters may also contain certain enhancer sequence elements that  
15 improve the efficiency of transcription.

The examples below describe the use of an expression vector that contains a kanamycin resistance gene as a selectable marker for selection of plants that have been transformed with the genetic construct. Numerous selectable markers, including antibiotic and herbicide resistance genes, are known in the art of plant molecular biology  
20 and may be used to construct expression vectors suitable for the practice of the present invention. Expression vectors may be engineered to include screenable markers, such as beta glucuronidase (GUS).

The genetic constructs employed in the examples below were engineered using the plasmid vector pART27 (Gleave, Plant Mol. Biol. 20:1203-1207, 1992). It is  
25 anticipated that other plasmid vectors or viral vectors, or other vectors that are known in the art of molecular biology, will be useful in the development of a construct that may be used to transform a plant to obtain expression of a MinD protein coding sequence. The creation of a genetic construct suitable for transformation using the *Agrobacterium* system is described, however, any transformation system for obtaining transgenic plants  
30 may be used. The construction of a vector and the adaptation of that vector to a particular transformation system are both within the ability of one skilled in the art.

The present invention also contemplates a method for altering the shape, size and/or number of plastids in a plant, relative to the wild type plant. The method comprises the steps of making a genetic construct comprising a MinD protein coding sequence and a promoter, not be natively associated with the sequence, transforming the  
5 plant with the genetic construct, and growing the transgenic plant so created as to allow expression of the genetic construct. The genetic construct as a transgene in the plant will change the size, shape and/or number of the plastids in the plant cells of the plant.

Alterations in plastid size, shape and/or number via genetic engineering of MinD expression in accordance with the present invention has the potential to result in  
10 improved productivity or increased vigor due to enhanced photosynthetic capacity and allow enhanced production of commercially important compounds that accumulate naturally or as a result of genetic engineering. The ability to alter the expression of the chloroplast division genes allows the manipulation of the size and number of chloroplasts in plant cells. Because chloroplast number is known to have a direct effect  
15 on photosynthetic capacity, it is likely that by manipulating levels of plastid division proteins in genetically engineered plants to achieve increased numbers or size of plastids, one may obtain plants having advantageous properties.

In the examples below, changes in chloroplast numbers and size were examined in plants in which a MinD protein coding sequence was expressed as a transgene in  
20 transgenic plants. It is expected that MinD protein coding sequences are also involved in regulating the division of other plastids, including chromoplasts, amyloplasts, and leucoplasts. These plastids are of great agronomic importance because they synthesize carotenoids, starch, and oils. Manipulation of the expression of chloroplast division sequences to alter the number or size of plastids other than chloroplasts is within the  
25 scope and spirit of the present invention.

Figure 2 sets forth a model for the structural organization of the plastid division apparatus in plants in which plastid division is mediated by two FtsZ-containing plastid-dividing rings, one localized on the stromal surface of the inner chloroplast envelope membrane containing FtsZ1, and the other on the cytosolic surface of the outer  
30 envelope membrane containing FtsZ2 (Figure 2, Panel A). Implied in this model is the coordinated positioning of division components across the envelope at the plastid

midpoint. In wild-type plants, both stromal and cytosolic PD rings, proposed to contain FtsZ1 and FtsZ2, respectively, are localized at the plastid midpoint, and the coordinated constriction of the two rings results in symmetric division yielding two daughter plastids approximately equal in size (Figure 2, panel A). Antisense repression of MinD leads to

5 misplacement of the stromal FtsZ1 ring in many, though not necessarily all, plastids. When it does occur, the cytosolic FtsZ2 ring in turn becomes localized to a site on the outer membrane corresponding to the site of misplacement of the FtsZ1 ring (Figure 2, panel B, right). Other components of the two PD rings presumably also assemble at this position. The coordinated action of the two mislocalized PD rings results in a productive

10 but asymmetric division event, yielding daughter plastids of unequal size. Multiple rounds of plastid division in which the stromal FtsZ1 ring was sometimes but not always misplaced could further increase the size variability.

The nonlimiting examples that follow are intended to be purely illustrative.

### EXAMPLES

#### 15 **Isolation and Characterization of a MinD gene in *Arabidopsis thaliana*.**

An homolog of the bacterial MinD gene was isolated from *Arabidopsis thaliana* as follows. The amino acid sequence of *Chlorella vulgaris* MinD was used as a query sequence to search the nonredundant GenBank database using the TBLASTN algorithm. A highly significant match was found to an open reading frame in the P1 library clone

20 MZF18 (accession number AB009056) from chromosome V of *Arabidopsis*. This *Arabidopsis* DNA sequence, designated *AtMinD1*, is shown in SEQ ID NO:1. It contains an open reading frame (ORF) spanning nucleotides 32,980 through 33,957 on the minus strand of MZF18, which is uninterrupted by introns and encodes a polypeptide of 326 amino acids with a calculated molecular weight of 35,690. This

25 *Arabidopsis* MinD amino acid sequence, designated AtMinD, is shown in SEQ ID NO:2.

Fig. 1 illustrates a sequence comparison by sequence alignment between the sequence from *Arabidopsis* and the corresponding gene sequences from other photosynthetic organisms. The sequences used in the alignment are identified as

30 follows: *Sy*, *Synechocystis* PCC6803, Q55900; *Gt*, *Guillardia theta* (plastid genome),

AAC35621; *Cv, Chlorella vulgaris* (plastid genome), P56346; *Pw, Prototheca wickerhamii* (plastid genome), CAB53105; *No, Nephroselmis olivacea* (plastid genome), AAD54908; *At, Arabidopsis thaliana*, AB009056 (translated sequence of nucleotides 32,980 through 33,957, minus strand); *Os, Oryza sativa* AF149810 (partial  
 5 sequence). Only the first 163 amino acids of the *O. sativa* sequence were used in the alignment. Dashes (-) indicate gaps in the alignment. Gaps at the amino termini were removed manually. Identical amino acids among the sequences shown are boxed in black. Asterisks (\*) indicate residues identical among all proteins when the following bacterial MinD sequences are added to the alignment (not shown): *Bacillus subtilis*,  
 10 Q01464; *Escherichia coli*, BAA36022; *Helicobacter pylori* 26695, AAD07400; *Deinococcus radiodurans*, AAF10331; *Aquifex aeolicus*, AAC06996.

This alignment of several MinD amino acid sequences in Fig. 1 from various photosynthetic organisms was performed using CLUSTAL W 1.8. The alignment revealed regions of high sequence similarity, indicating that the gene has been highly  
 15 conserved during the evolution of chloroplasts. The MinD protein encoded by *AtMinD1* shares a 65% identity with the MinD protein from *Chlorella vulgaris* (P56346), a slightly lower extent of identity (58-62%) with the MinD proteins encoded in the plastid genomes of *Guillardia theta* (AAC35621), *Prototheca wickerhamii* (CAB53105), *Nephroselmis olivacea* (AAD54908) and *Oryza sativa* (AF149810), and a 53% identity  
 20 with the MinD protein from the photosynthetic prokaryote *Synechocystis* (PCC6803, Q55900). *AtMinD* also shares a greater than 40% amino acid identity with the bacterial MinD sequences of *Bacillus subtilis* (Q01464); *Escherichia coli* (BAA36022); *Helicobacter pylori* 26695 (AAD07400); *Deinococcus radiodurans* (AAF10331); and *Aquifex aeolicus* (AAC06996), data not shown. Sequence identity was calculated using  
 25 the SIM local alignment algorithm (Huang and Miller 1991) with the default parameters specified on the ExPASy Molecular Biology Server, Swiss Institute of BioInformatics (<http://expasy.hcuge/sprot/sim-prot.html>).

#### **Isolation and Characterization of a MinD gene in *Tagetes erecta* (Marigold).**

An homolog of the bacterial MinD gene was isolated from *Tagetes erecta*  
 30 (Marigold) as follows. Color complementation was used to screen a cDNA library made

from poly A RNA isolated from stage 3 and 4 petals (approximately 8-10 mm and 13-15 mm in length, respectively) taken from the marigold variety Dark Orange Lady (W.

Atlee Burpee Company, Clinton, Iowa). This technique relies on the ability of an *E. coli* engineered to express certain carotenoids to accumulate the carotenoids when carotenoid

5 biosynthetic genes are expressed from a plasmid. A second plasmid from a library of interest can be introduced into this background with a different selectable marker and a compatible replicon which enables the selection of colonies having a desired phenotype. This color complementation method has proven to be effective for isolating carotenoid biosynthetic genes from a number of organisms.

10 To construct the cDNA library, total RNA from the marigold petals was isolated by LiCl precipitation and poly A RNA was obtained by two passes of the RNA over an oligo dT cellulose column. (Stratagene, La Jolla, CA). Single clone excision and mass excision of the lambda library to yield phagemids were then performed as recommended by the manufacturer. Isolated plasmids were sequenced by primer walking on both  
15 strands using a dRhodamin cycle sequencing kit.

*E. coli* cells (strain DH5 $\alpha$ ) containing the plasmid pAC-ZEAX for producing zeaxanthin were transformed with plasmids containing cDNA from the Marigold library, and then grown in LB supplemented with chloramphenicol to maintain the carotenoid-gene containing plasmid and with ampicillin (100  $\mu$ g/ml) to maintain the  
20 marigold library plasmids.

A low temperature screen led to the isolation of a marigold gene encoding the plastid division protein MinD. This screen was based on the observation that *E. coli* genetically engineered to accumulate zeaxanthin grew more slowly at 18°C than *E. coli* harboring the vector plasmid alone and accumulated significantly less zeaxanthin than  
25 when grown at 37°C. The basis of this temperature dependent phenotype is unknown. When the marigold cDNA library was transformed into zeaxanthin containing *E. coli*, numerous rapidly growing, highly pigmented colonies were identified in a background of pale, slow growing colonies. Plasmids isolated from several of these colonies were sequenced and similarity searches against the publicly available databases revealed a  
30 marigold gene with similarity to the *E. coli* MinD protein. This *Tagetes* DNA sequence, designated *TeMinD*, is shown in SEQ ID NO:3 and its deduced amino acid sequence,

designated TeMinD, is shown in SEQ ID NO:4.

### Construction of Chimeric Sense and Antisense MinD Protein Coding Sequences.

The MZF18 clone (AB009056) was obtained from the *Arabidopsis* Biological Research Center in Columbus, Ohio. The region corresponding to the *AtminD1* ORF  
5 was amplified from MZF18 with Deep Vent Polymerase (New England BioLab) using the following primers: forward primer,  
5'-CCGAATTCGAAGCAGCAGCACTATCAATGG-3'; reverse primer  
5'-CGGAATTCGATCCGTTTGCCATTAGCC-3'. Both primers incorporated recognition sites for *EcoRI*. The PCR product was sequenced in its entirety to ensure  
10 that no mutations had been introduced, and ligated in both orientations into pBluescript (Stratagene). The plasmid with the 5' end of the insert nearest the T3 promoter was designated KG405; the plasmid with 5' end of the insert nearest the T7 promoter was designated KG406. The plasmids were maintained in a *minCDE* deletion strain of *E. coli*, RC3F. For the transgenic constructs, the *EcoRI*-restricted PCR fragment was  
15 ligated into the *EcoRI* cloning site of pART7 behind the CaMV 35S promoter in either the sense or antisense orientation. The transgenes were then excised from the resulting plasmids with *NotI* and ligated into the *NotI* cloning site in the binary transformation vector pART27, yielding plasmids KG402 containing the *AtMinD1* antisense construct, and KG404 containing the *AtMinD1* sense construct. Both transformation vectors also  
20 included a selectable marker from pART27 conferring plant resistance to kanamycin.

### Characterization of MinD Synthesis in Plants.

Relative to the MinD proteins from *C. vulgaris* and several prokaryotes, AtMinD contains an amino terminal extension with features common to chloroplast transit peptides. These include alanine as the second residue, a relatively high proportion of  
25 hydroxylated amino acids, and few acidic residues. An in vitro chloroplast import assay was performed to determine whether this extension was able to function as a chloroplast targeting sequence.

Plasmid KG405, described above, was linearized with BamHI and transcribed

using T3 RNA polymerase. A plasmid containing the prSS control encoding the small subunit of pea RuBP carboxylase was linearized with *Pst*I and transcribed with SP6 RNA polymerase. The resulting transcripts were translated in a rabbit reticulocyte lysate translation system (Promega) containing [<sup>35</sup>S]methionine (DuPont/NEN). Import reactions were carried out using chloroplasts isolated from 8- to 12-day-old pea seedlings (*Pisum sativum* var. *Little Marvel*, Olds Seed Company, Madison, WI) and purified over a Percoll gradient. Intact chloroplasts were reisolated and resuspended in import buffer (330 mM sorbitol, 50 mM Hepes/KOH, pH 8.0) at a concentration of 1 mg chlorophyll/ml. Thermolysin treatment of import products was performed and import products were analyzed by SDS-PAGE and fluorography.

The in vitro transcription of the *AtMinD1* ORF, followed by the in vitro translation of the resulting transcript in the presence of [<sup>35</sup>S]methionine, yielded a full-length, radiolabeled translation product that migrated at 39.7 kD, somewhat above its calculated mass. When added to the isolated pea chloroplasts, the translation product was processed to a smaller form migrating at 35.6 kD. The processed form of the protein was soluble following import, and was fully protected from a post-import treatment with the protease thermolysin. In a control set of reactions, the small subunit of pea RuBP carboxylase/oxygenase, a soluble stromal protein, behaved identically. These results provide strong evidence that *AtMinD*, like the *Arabidopsis* FtsZ1, is synthesized as a precursor on cytosolic ribosomes and posttranslationally targeted to the chloroplast where it is processed to a mature form.

#### **Effect of Antisense Repression of MinD on Chloroplast Size and Number.**

To demonstrate that the *AtMinD1* gene functions in the placement of the plastid-localized FtsZ ring and the positioning of the plastid division machinery, the transformation vector KG402, containing the *AtMinD1* antisense construct, was introduced into *Arabidopsis* plants (ecotype Columbia (Col-0)) by *Agrobacterium*-mediated transformation.

The *Arabidopsis* plants utilized arose from seeds sown on a Supersoil potting mix and vermiculite in a ratio of 3:1. The seeds were incubated at 4°C in the dark for two days before being moved to growth chambers and grown at 22°C with 16 hrs of

daylight. The age of the plants was calculated from the first day of their transfer to growth chambers.

*Agrobacterium*-mediated transformation was performed by a freeze-thaw method using *Agrobacterium tumefaciens* C58 (GV3101). The plasmids were checked for  
5 rearrangements following transfer to *Agrobacterium* by back-transformation to *E. coli* and restriction analysis. The transformation vectors were introduced into the *Arabidopsis* plants using the floral dip procedure. Transformants were selected by germination in nutrient medium containing 50 or 100 mg/l kanamycin and transplanted to soil 7-10 d after germination for propagation and analysis. Kanamycin resistant  
10 (kan<sup>r</sup>) plants that originated from different pots were assumed to be derived from independent T-DNA insertion events for the purposes of phenotype characterization.

T<sub>1</sub> seeds were harvested from the inoculated plants, and transformants were selected on the basis of their resistance to the antibiotic. Leaf tissue from kan<sup>r</sup> plants were examined microscopically for effects on chloroplast size and number. Plants from  
15 19 different pots were analyzed, ensuring that the phenotypes observed were the result of a minimum of 19 independent transformation events. Based on recent studies of T-DNA insertion patterns in *Arabidopsis* transformed by a similar procedure, it is likely that most of the kan<sup>r</sup> T<sub>1</sub> individuals, including those originating from the same pot, represented independent insertion events.

20 The phenotypes of the antisense transformants were initially investigated by examination of mesophyll cells from first leaves of 23-day-old T<sub>1</sub> plants. In wild-type plants, the leaves at this stage of development are fully expanded such that the cells have accumulated their full complement of approximately 100 chloroplasts, all of which fall within a narrow range of sizes (Figure 3). Among the 164 kan<sup>r</sup> individuals examined  
25 from the 19 antisense transformations, 90 (55%) exhibited phenotypes that differed noticeably from wild type. Among these, 66 (73%) displayed a striking degree of heterogeneity in the sizes of the chloroplasts within a single mesophyll cell. This heterogeneity was evident both from visual inspection of the mesophyll cells under the microscope and from measurements of the frequency with which chloroplasts of  
30 different sizes were observed in the same cell (Figure 3, panels B-D). The plastid size heterogeneity was even more pronounced in smaller cells from younger leaves in which



chloroplasts are not yet fully expanded as they are in 23-day-old leaves.

Chloroplast numbers per unit cell area were also quite variable in the antisense plants, in contrast with wild type in which the number of chloroplasts per cell is tightly correlated with cell size. However, the chloroplasts were consistently fewer in number and larger in size than in wild type cells, suggesting a reduced number of plastid division events in most of the *AtMinD1* antisense lines. The phenotypes observed in the T<sub>1</sub> generation were also observed in T<sub>2</sub> and T<sub>3</sub> progeny.

Although chloroplast numbers in cells from antisense plants were consistently lower and the chloroplast sizes far less uniform than in wild type, the linear relationship between the total chloroplast plan area and the total mesophyll cell plan area in the antisense lines was approximately the same as in wild type. This finding indicates that the reduced chloroplast numbers were compensated for by corresponding increases in chloroplast expansion so that total chloroplast volume was conserved. Similar results have been shown for other perturbations in chloroplast number and/or expansion.

A relatively small proportion (18%) of T<sub>1</sub> plants with visually detectable phenotypes under microscope displayed less heterogeneity in chloroplast size within single cells. Instead, the mesophyll tissue in these plants comprised a mixture of cells containing either wild-type numbers and sizes of chloroplasts or only a few large chloroplasts. Because the affected cells contained fewer chloroplasts than the number of proplastids present in leaf primordia, these observations suggest a significant inhibition of both proplastid and chloroplast division in some cells, but not in others.

Under the growth conditions used for the experiments, plants expressing the *AtMinD1* antisense transgene grew more rapidly than wild type in the early stages of development (first leaves appeared earlier), but inflorescences appeared a few days later. This difference was evident through the T<sub>2</sub> and T<sub>3</sub> generations. In other aspects of growth and development, the antisense plants did not differ noticeably from wild type, however, careful measurements of growth parameters may reveal other subtle differences.

To confirm that the transgenic phenotypes resulted from reduced expression of the endogenous *AtminD1* gene, a northern blot of poly(A)<sup>+</sup> RNA isolated from antisense and wild type plants was probed with a radiolabeled RNA probe specific for *AtMinD1*.

Total RNA was isolated from 23-27-day-old plants as described previously using 1 g of leaf tissue from independent transgenic lines (T<sub>3</sub>) or from wild type to determine the expression levels of the *AtMinD* protein coding sequence. Only transgenic individuals exhibiting plastid size heterogeneity for the *AtMinD1* antisense plants were used for

5 RNA isolation. Poly(A)<sup>+</sup> RNA was isolated with Oligotex resin (Qiagen) according to the manufacturer using total RNA as starting material, and quantified by measuring absorbance at 260 and 280 nm. Poly(A)<sup>+</sup> RNA gel blots were prepared as described previously using nylon membrane (Micron Separations, Inc.). An RNA probe for hybridization specifically to sense *AtMinD1* mRNA was prepared by linearizing KG406

10 with HindIII, and carrying out an in vitro transcription reaction in the presence of <sup>32</sup>P-UTP (800 Ci/mmol; ICN, Costa Mesa, CA) as described previously, but using T7 RNA polymerase (New England BioLab). Blots were hybridized overnight and washed in 0.2X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 68°C.

The probe hybridized to two transcripts of about 1.1 and 1.7 kb, the smaller of

15 which was more abundant. The probe remained bound to both transcripts when the blot was washed at very high stringency, indicating the two mRNAs were derived from either the same gene or from two closely related genes. Based on the size of the *AtMinD1* open reading frame (978 bp), it was expected that *AtMinD1* is represented by at least the smaller transcript. The levels of both transcripts were significantly reduced

20 in the antisense plants when compared with wild type, indicating that the heterogeneity in chloroplast size and number in these plants was the result of reduced *AtMinD1* expression.

To determine whether the heterogeneity in chloroplast size observed in the antisense repression of *AtMinD1* could be the result of asymmetric chloroplast division,

25 petal tissue from flowers of the transgenic plants were examined. Normally, in leaves of dicotyledonous plants, the division of chloroplasts is rapid and is not synchronized. Consequently, it can be difficult to observe chloroplasts in the process of division, particularly in the *AtMinD1* antisense plants in which chloroplast numbers are reduced. However, in the present case, a high frequency of constricted plastids were documented

30 in the *Arabidopsis* petals and easily viewed because the plastids were less densely packed than in mesophyll cells.

In many of the constricted plastids, the constriction was noticeably displaced from the center. This is in marked contrast from wild type plants in which petal plastids almost always appear to be constricted in the center. Asymmetric constriction of chloroplasts in leaf epidermal cells in the transgenic lines was also observed.

- 5 Collectively, this data suggests that the chloroplast size variability in the *AtMinD1* antisense plants results at least partially from asymmetric plastid division.

#### **Effect of MinD Overexpression in Transgenic Plants.**

- To further analyze the role of *AtMinD1* in plastid division, the transformation vector KG404, containing the sense *AtminD1* construct under control of the CaMV 35S  
10 promoter, was introduced into *Arabidopsis* plants by *Agrobacterium*-mediated transformation as described above.

- The phenotypes of 82 kan<sup>r</sup> T<sub>1</sub> individuals representing at least 13 independent transformation events were investigated microscopically. The predominant phenotype, observed in 52 (73%) of the 71 T<sub>1</sub> plants having phenotypes that were clearly  
15 distinguishable from wild type, was a dramatically reduced number of greatly enlarged chloroplasts in comparison to wild type. Cells in most of these plants appeared to contain five or fewer chloroplasts, and many had only a single large chloroplast. This phenotype contrasted with that observed in most of the antisense plants, in which the chloroplasts were generally more numerous, and indicates a more severe inhibition of  
20 plastid division. The phenotype was also inherited in the T<sub>2</sub> and T<sub>3</sub> progeny. Because the number of chloroplasts in mesophyll cells from the *AtMinD1* overexpression lines was less than the number of proplastids present in the cells of the shoot apical meristem, it is believed that a disruption of both proplastid and chloroplast division in these plants occurred.

- 25 To confirm that the transgenic phenotypes resulted from overexpression of the endogenous *AtminD1* gene, a northern blot of poly(A)<sup>+</sup> RNA isolated from sense and wild type plants was performed using a radiolabeled RNA probe specific for *AtMinD1*. Total RNA was isolated from 23- to 27-day-old plants as described previously using 1 g of leaf tissue from independent transgenic lines (T<sub>3</sub>) or from wild type to determine the  
30 expression levels of the *AtMinD* protein coding sequence. Only transgenic individuals

exhibiting severely reduced numbers of chloroplasts for the *AtMinD1* sense plants were used for RNA isolation. Northern blot analysis confirmed that the severe disruption in chloroplast division was accompanied by *AtminD1* overexpression.

The remaining 19 (29%) T<sub>1</sub> individuals among the 71 that differed obviously  
5 from wild type had less severe defects in plastid division. Most of these resembled the antisense plants, having variable numbers and sizes of chloroplasts. It has not been determined whether this phenotype is indeed the result of *AtMinD1* overexpression, although it parallels findings in *E. coli* that suggest overexpression of *minD* induces minicell formation. However, this phenotype is also consistent with cosuppression of  
10 endogenous *AtMinD1* gene expression. The *AtMinD1* sense lines grew somewhat more slowly and did not grow as large as the wild type or antisense plants. They also began flowering about 3 days earlier on average.

Taken together, these results indicate that either decreased or increased numbers of chloroplasts can be obtained in transgenic plants by manipulation of MinD expression  
15 levels. Manipulation of the size and shape of chloroplasts may also be obtained.